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Viability and niche segregation of *Prochlorococcus* and *Synechococcus* cells across the Central Atlantic Ocean

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ABSTRACT: A membrane permeability test (cell digestion assay) was used to quantify the proportion of living cells (%LC) in the natural populations of *Synechococcus* and *Prochlorococcus* across the Central Atlantic Ocean. %LC varied greatly from 48 to 100% for *Prochlorococcus* and from 15 to 100% for *Synechococcus*. The %LC was smaller in surface (5 to 30 m) waters of the South Atlantic subtropical gyre, where lower values were observed for *Prochlorococcus* (64 ± 4%, mean ± SE) than for *Synechococcus* (74 ± 6%). The %LC increased in the North Atlantic. This is probably associated with the nutrient enrichment by the equatorial upwelling, with *Synechococcus* showing a more significant response than *Prochlorococcus*. The vertical distribution of %LC differed between the 2 picocyanobacteria groups, with the maximal %LC of *Synechococcus* found at shallower depths (i.e. those receiving 25% light) than that of *Prochlorococcus*. *Prochlorococcus* showed increased viability as light decreased, suggesting a lower resistance to high light and probably a greater capacity to survive at low irradiance than *Synechococcus*. These contrasting vertical and spatial distributions of picocyanobacteria %LC suggest that these organisms have differing optimal niches in the Central Atlantic, segregated by their different sensitivity to stress conditions. The results indicate that *Prochlorococcus* experiences higher cell death than *Synechococcus* in the Central Atlantic Ocean. This study identifies cell death as a process structuring phytoplankton communities, which is likely to affect the carbon flow in the Central Atlantic Ocean.

KEY WORDS: Phytoplankton · *Synechococcus* · *Prochlorococcus* · Atlantic Ocean · Cell death · Light

INTRODUCTION

Recent reports of high phytoplankton lysis rates identify phytoplankton cell death as an important loss process in marine waters (van Boekel et al. 1992, Brussaard et al. 1995, Agustí et al. 1998, Agustí & Duarte 2000). The high phytoplankton lysis rates reported for oligotrophic waters (Agustí et al. 1998, 2001, Agustí & Duarte 2000) imply that an important proportion of phytoplankton cells may be dead in these communities, which are dominated by the picocyanobacteria *Synechococcus* and *Prochlorococcus* (e.g. Waterbury et al. 1986, Olson et al. 1990, Partensky et al. 1999). The death and subsequent lysis of *Synechococcus* and *Prochlorococcus* cells in the oligotrophic ocean presumably result in the release of the carbon incorporated by photosynthesis as dissolved organic carbon. This may have important consequences for the carbon flux in the oligotrophic ocean, stressing the need to address the status of the dominant picocyanobacteria.

Information on the status of phytoplankton cells in natural communities is, however, very scarce, due to methodological difficulties in discriminating dying from healthy (or living) phytoplankton cells in nature. The recent introduction of new techniques for the identification of living and dying cells in natural phytoplankton communities (Veldhuis et al. 2001, Agustí & Sánchez 2002) now allows quantification of the abundance of living and dead cells within these phytoplankton communities. These studies reported that, in

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the temperate waters examined, the proportion of dead cells within natural marine phytoplankton communities could be considerable (Veldhuis et al. 2001, Agustí & Sánchez 2002).

The goal of this study was to test the hypothesized importance of cell death for populations of *Synechococcus* and *Prochlorococcus* in the oligotrophic ocean by quantifying the proportion of living versus dead cells in the populations across the Central Atlantic Ocean. Living *Synechococcus* and *Prochlorococcus* cells were quantified using the cell digestion assay (Agustí & Sánchez 2002), a membrane permeability test that removes dead cells (which show increased membrane permeability) from the sample, thereby allowing the unambiguous identification of living cells in natural communities. The spatial and vertical variability in the percentages of living cells was analyzed to identify patterns in the distribution of dead cells in the Central Atlantic Ocean, and to distinguish any consistent differences between *Synechococcus* and *Prochlorococcus.*

MATERIALS AND METHODS

The study was performed during the LATITUDE-4 cruise across the Central Atlantic Ocean, on board the spanish RV 'BIO-Hespérides'. The cruise started in Rio de Janeiro on March 16, 2000, and ended on April 4, 2000, at the Canary archipelago. A total of 14 stations distributed along the South Atlantic subtropical gyre, the inter-tropical zone and the eastern North Atlantic were occupied (Fig. 1). Seawater samples were collected in Niskin bottles attached to a Rossette-CTD system, at an average of 12 depths from the surface to 200–300 m.

Underwater light (photosynthetically active radiation, PAR) was measured at each station from the surface to 200 m depth, using a 4π underwater quantum sensor (LI-192SA) attached to a SATLANTIC-OCP 100 automatic profiler. The percentage of light received at the different sampling depths was calculated relative to the light received at 0.5 m depth.

The abundances of *Prochlorococcus* and *Synechococcus* were quantified in replicated fresh samples using a FACSCALIBUR (Becton-Dickinson) flow cytometer, fitted with a 488 nm laser and a photomultiplier for detection of forward scattered light. An aliquot of a calibrated solution of 1 µm diameter high-green fluorescent beads (Polysciences) was added to the samples as an internal standard for the quantification of cell concentration. Bead concentration in the standard solution was calculated by filtering replicated aliquots onto black nuclepore filters and counting the beads under an epifluorescence microscope. The red, green and orange fluorescence emissions, and the forward and side scattering of the cells and beads, were used to detect different cell populations and to differentiate them from the fluorescent beads.

The abundance of living picocyanobacteria cells was analyzed throughout the water column in 9 of the 14 stations sampled (Fig. 1) using a cell membrane permeability test (cell digestion assay, Darzynkiewicz et al. 1994), as modified for phytoplankton cells by Agustí & Sánchez (2002). The cell digestion assay is based on brief exposure to the enzymes Trypsin and DNAse I, which enter the cytoplasm of cells with damaged plasma membranes (i.e. necrotic or advanced apoptotic cells) resulting in their entire digestion, while having little or no effect on the viability, morphology or function of live cells (Darzynkiewicz et al. 1994, Agustí & Sánchez 2002). Digestion of dead cells consists of fragmentation and hydrolysis of DNA by DNAse I, and peptide hydrolysis by Trypsin. The digested dead cells are undetectable by optical observation and lose any fluorescent signals (both autofluorescence and staining), and are, therefore, effectively removed from the population (Darzynkiewicz et al. 1994, Agustí & Sánchez 2002).

Stock solutions of DNAse I (400 µg ml^{-1}) and Trypsin (1%) were prepared in Hanks' balanced salt solution

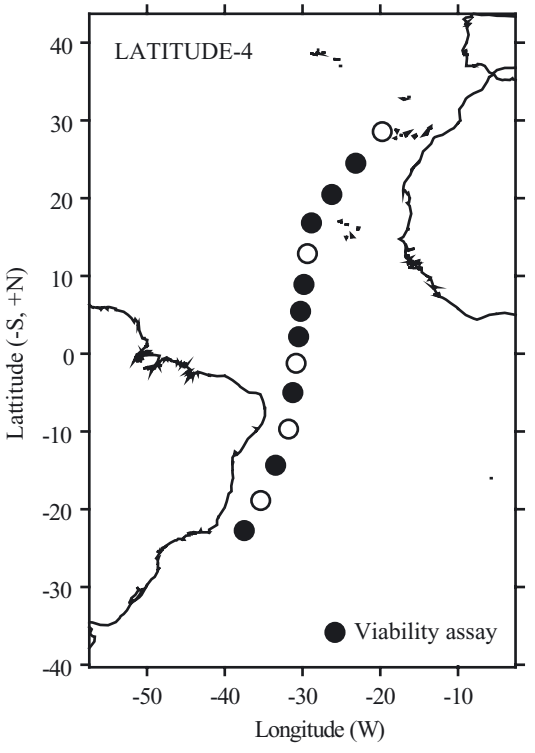

Fig. 1. Position of the stations sampled during the LATITUDE-4 cruise (●, ○). Solid circles identify the stations where the enzymatic cell digestion assay was performed

(HBSS) (Sigma) and kept frozen at –65°C until use. Viability tests were made on replicated 1 ml fresh seawater samples by adding 200 µl of DNAse I stock solution and incubating for 15 min at 36°C, and then adding 200 µl of Trypsin solution and incubating for an additional 30 min at 36°C (Darzynkiewicz et al. 1994). At the end of the incubation the samples were placed on ice to stop the enzyme reaction, and the abundance of picocyanobacteria cells remaining was quantified using a flow cytometer, as described above. Tests confirmed that neither the 45 min incubation nor the procedure used to stop the enzyme reaction (i.e. placing the samples on ice) affected cell abundance. No significant differences were found in the abundances of *Synechococcus* and *Prochlorococcus* (quantified in replicated samples) between untreated samples and samples incubated at 36°C for 45 min without enzymes ($1.51 \times 10^5 \pm 1.9 \times 10^3$ and $1.49 \times 10^5 \pm 5.6 \times 10^3$ cells ml^{-1}, and $1.5 \times 10^3 \pm 42$ and $1.6 \times 10^3 \pm 89$ cells ml^{-1}, before and after incubation for *Prochlorococcus* and *Synechococcus*, respectively).

The cells remaining after the enzymatic treatment, i.e. those having intact membranes, were considered to represent living or viable cells. Dead cells, with compromised membranes, were digested by the enzymatic cocktail and were undetectable by the flow cytometer. The fraction of living picocyanobacteria cells in the samples was calculated by dividing the concentration of living cells after the enzyme treatment by the cell concentration in the untreated sample, representing the total (dead plus living) cell concentration. The number of dead cells was calculated by subtracting the number of living cells from the total cell abundance.

The accuracy of the cell digestion assay in quantifying picocyanobacteria viability was tested by comparing the percentage of living cells (%LC) of a *Synechococcus* sp. (CCMP833) culture quantified by the cell digestion assay against the results obtained using a vital stain (Bac-light Kit, Molecular Probes). The Bac-light Kit is a membrane permeability test based on a double staining: dead cells are stained with propidium iodide (PI) and fluoresce red, and living cells are stained by SYTO 9 and fluoresce green under blue light illumination. The Bac-light kit cannot be used to assess viability in natural picoplankton communities because the resulting fluorescence masks chlorophyll *a* autofluorescence, precluding discrimination between autotrophic and heterotrophic picoplankton. A batch culture of *Synechococcus* sp. (CCMP833), growing at 21°C in f/2 medium under continuous illumination, was sampled during the growing and stationary phases. Duplicated samples from the culture were incubated with DNAse and Trypsin at 36°C, as indicated above, with duplicated samples incubated as blanks. After the incubation, the samples were quantified using a flow cytometer (as indicated above). Duplicated 1 ml samples were also stained with 0.1 µl of the Bac-light Kit, following Lee & Rhee (1997), and filtered onto 0.2 µm black nuclepore filters. The filters were examined under an epifluorescence microscope (Zeiss Axioplan Imaging) to quantify living (green fluorescent) and dead (red fluorescent) cells. The %LC obtained using both methods were in excellent agreement (Table 1), indicating that the results derived from the cell digestion assay are perfectly comparable with those obtained using another cell membrane permeability test and, therefore, that this assay correctly quantifies the abundance of living picocyanobacteria cells.

RESULTS

The area sampled included (1) the warm and permanently stratified waters of the South Atlantic subtropical gyre (SASG), where surface (5 m) temperatures reached a maximum of 28.54°C and nutrients were depleted down to a depth of 150 m (Fig. 2); (2) the intertropical zone (ITZ), where the thermocline shallower and subsurface waters were nutrient enriched as a result of the equatorial upwelling, which is located north of the equator (Fig. 2); and (3) the oligotrophic waters of the eastern North Atlantic (ENA), where the thermocline deepened again (Fig. 2). The SASG was the clearest and most oligotrophic area sampled, showing the lowest chlorophyll *a* concentration (0.018 mg m^{-3} at 5 m depth) and a deep chlorophyll *a* maximum (DCM) located at around 140 m depth.

The picocyanobacteria cells remaining after the cell digestion assay showed similar autofluorescence signals (red and orange fluorescence) in the flow cytometer, as well as similar side scattering signals, to those observed for control samples, allowing the unambiguous identification of living *Prochlorococcus* and *Synechococcus* cells after the enzymatic digestion of dead cells. The analysis was made using fresh samples which yielded sufficient red fluorescence signals from surface *Prochlorococcus* cells to allow their detection by flow cytometry. Although picoeukaryotic cells were readily identified by flow cytometry, they were present

Table 1. *Synechococcus* sp. (CCMP833). Percentage of living cells (%LC), obtained using the cell digestion assay and the double vital stain Bac-light Kit (Molecular Probes)

Cell digestion assay	Bac-light Kit
96 ± 1.6	99 ± 0.5
80 ± 4.7	75 ± 5.1
24 ± 1.0	26.5 ± 1.3

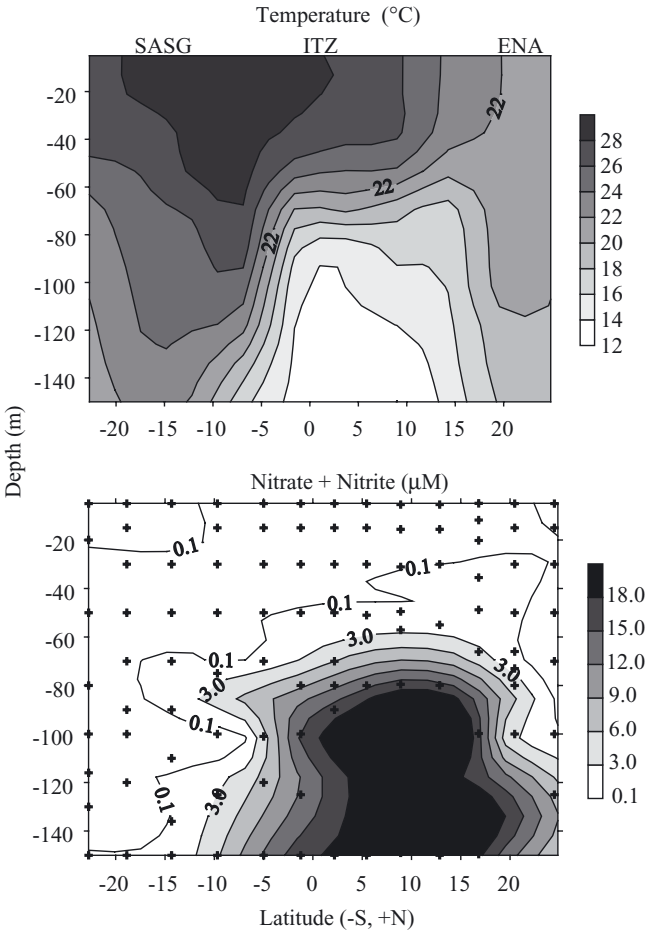

Fig. 2. Distribution of temperature (°C) and nitrate + nitrite concentration (µM) in the Atlantic Ocean during the study. SASG = South Atlantic subtropical gyre, ITZ = inter-tropical zone, ENA = eastern North Atlantic

at low abundance at most stations, precluding the quantification of living eukaryotic cells in the study.

Prochlorococcus was more abundant than *Synechococcus*, with average (±SE) concentrations of 1.0×10^5 ($\pm 7.1 \times 10^3$) and 5.0×10^3 ($\pm 8.6 \times 10^2$) cells ml^{-1}, respectively. The abundance of *Synechococcus* varied greatly along the transect, being lowest in the SASG waters and highest in the North Atlantic waters, particularly in the area influenced by the equatorial upwelling (Fig. 3). *Prochlorococcus* was also more abundant in the North Atlantic, although the variability was smaller than that observed for *Synechococcus* (Fig. 3). *Prochlorococcus* abundance extended to deeper waters than that of *Synechococcus* (Fig. 3).

The %LC varied from 48 to 100% for *Prochlorococcus* and from 15 to 100% for *Synechococcus*, averaging 78 ± 2 and 83 ± 2% (mean ± SE), respectively. The lowest %LC of *Prochlorococcus* was observed in surface waters (5 m), in contrast to *Synechococcus*, which had a minimum %LC in deep layers. The %LC of both *Synechococcus* and *Prochlorococcus* was lower in the South Atlantic, especially in the SASG waters, pointing to high picocyanobacteria cell death in this area. Here, lower %LC values were observed for *Prochlorococcus* (64 ± 5%) than for *Synechococcus* (74 ± 6%) in surface (5 to 30 m depth) waters (Fig. 4). Two peaks in the vertical distributions of %LC were observed in the SASG. The position of these %LC maxima in the water column differed, however, between the 2 groups, with the maxima of *Prochlorococcus* %LC located deeper (60 to 70 and 100 to 120 m, Fig. 4) than those of *Synechococcus* (20 to 30 and 80 to 90 m, Fig. 4). The %LC of picocyanobacteria increased, however, in the North Atlantic waters, especially in the area influenced by the equatorial upwelling (Fig. 4). Here the %LC at the surface (5 to 30 m depth) was higher for *Synechococcus* (85 ± 3%) than for *Prochlorococcus* (70 ± 2.5%). In the North Atlantic, values of %LC in excess of 90% were observed in deep waters for both groups, although the healthy *Synechococcus* cells extended over a wider area, with higher %LC at shallower depths than *Prochlorococcus* (Fig. 4). The large increase in the

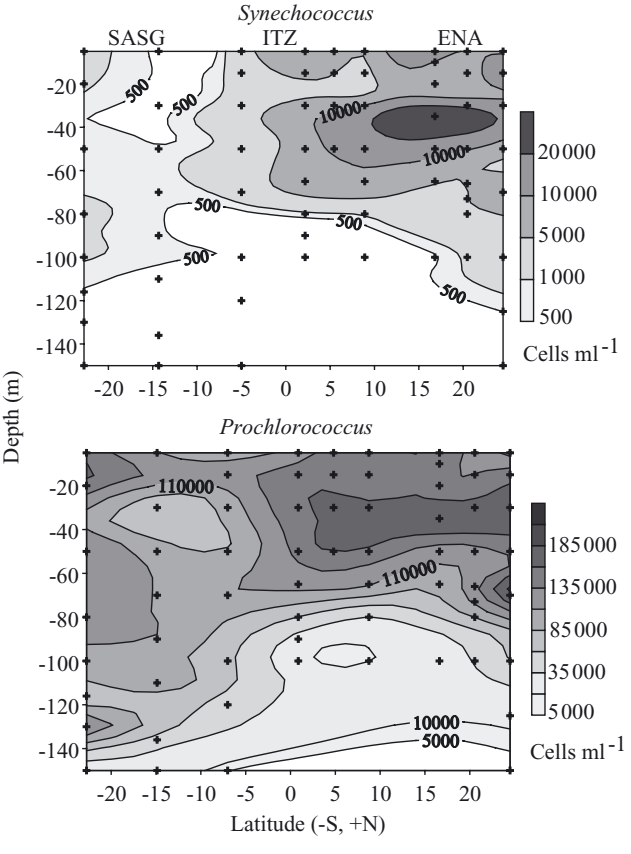

Fig. 3. *Synechococcus* and *Prochlorococcus*. Cell abundance distribution across the transect

proportion of dead *Synechococcus* cells with depth, indicated by the strong reduction in %LC, was not observed for *Prochlorococcus* (Fig. 4).

The examination of the mean %LC at different light levels revealed important differences between *Prochlorococcus* and *Synechococcus* cell viability with increasing irradiance. *Synechococcus* showed a parabolic distribution of %LC with light, with the highest average %LC of 95 ± 4% (i.e. lowest % dead cells) at depths receiving on average 25% light (relative to the light at 0.5 m) and the %LC decreasing with both increasing and decreasing light, reaching average values of 75 ± 4 and 78 ± 8% at higher (>40%) and lower (<1%) incident light, respectively (Fig. 5). In contrast, the %LC of *Prochlorococcus* showed a negative linear relationship with light, with the lowest fraction of living cells (64 ± 4%) at depths receiving more than 40% light, and the fraction increasing with decreasing light intensity to reach the highest average %LC of 91 ± 3% (mean ± SE) at depths receiving <1% light (Fig. 5).

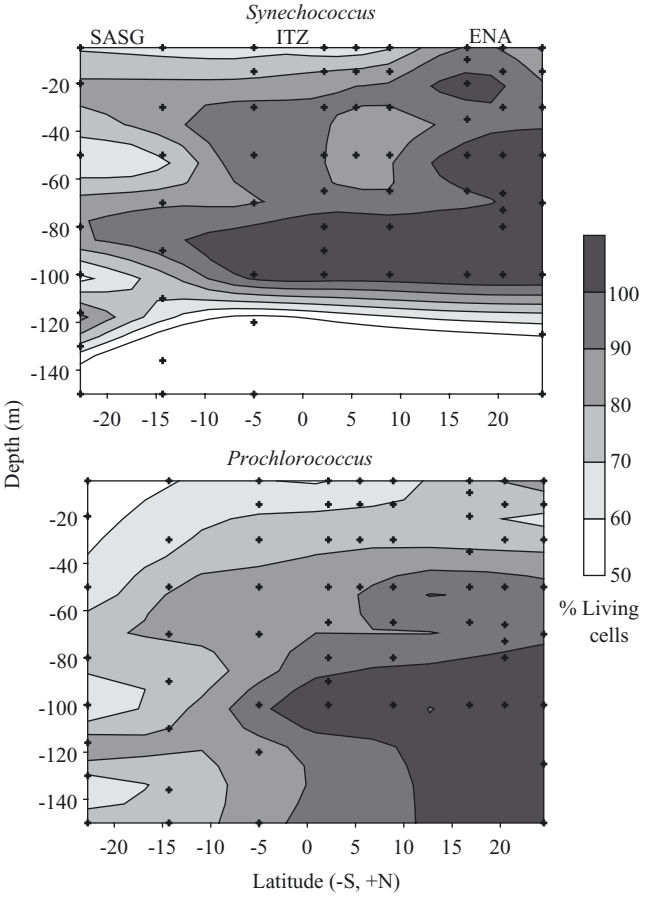

Fig. 4. *Synechococcus* and *Prochlorococcus*. Vertical and latitudinal distribution of the percentages of living cells across the Central Atlantic Ocean

DISCUSSION

The results presented reveal the presence of a significant fraction of dead picocyanobacteria cells in the Central Atlantic Ocean, suggesting that cell death must be an important process regulating picocyanobacteria dynamics in these waters, as concluded by Agustí et al. (2001). The %LC in both *Synechococcus* and *Prochlorococcus* populations showed high variability across the Central Atlantic Ocean, but this is comparable to values reported for natural phytoplankton communities in temperate waters (Veldhuis et al. 2001, Agustí & Sánchez 2002). Veldhuis et al. (2001) found %LC ranging from 40 to 95% for small phytoplankton cells in the North Atlantic (40° N, 23° W), with the %LC of *Synechococcus* ranging from 75 to 95%. A similar range of variation in the proportion of living phytoplankton cells was found in the Mediterranean littoral, where the %LC for *Synechococcus* was low (35%) at the beginning of the summer, but reached values of 100% during the summer bloom (Agustí & Sánchez 2002). There are, however, no previous reports on the fraction of living versus dead cells for natural *Prochlorococcus* populations.

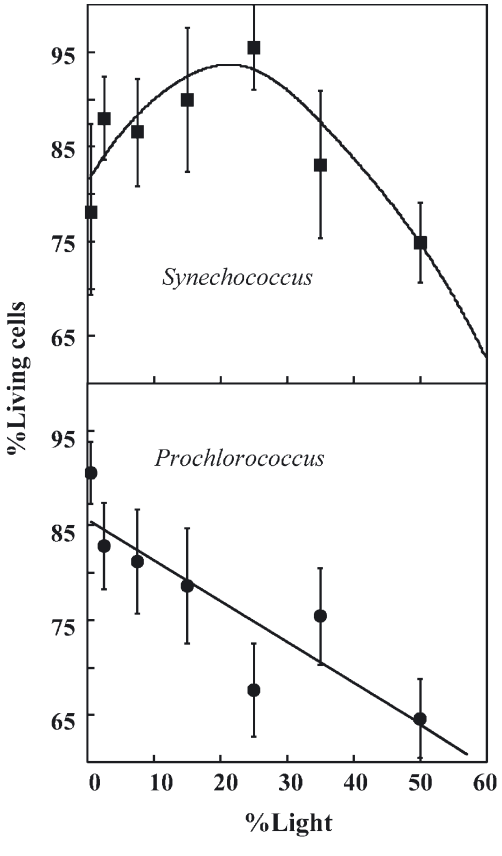

Fig. 5. *Synechococcus* and *Prochlorococcus*. Average (±SE) percentage of living cells in populations against relative incident light intensity (% light, PAR) throughout the water column in the Atlantic Ocean

The distribution of the proportion of living picocyanobacteria cells across the Central Atlantic Ocean reflected the trophic conditions of the waters. The %LC was lowest for both groups in the ultraoligotrophic waters of the SASG, indicating strong picocyanobacteria cell death in this area, while the highest %LC values were observed in the equatorial North Atlantic, associated with nutrient enrichment by the equatorial upwelling. Nutrient deficiency has been described to directly affect phytoplankton cell death in cultures (Brussaard et al. 1997, Lee & Rhee 1997, Berges & Falkowski 1998, Brussaard & Riegman 1998). Both the abundance of cells and the %LC for *Synechococcus* increased greatly in the area influenced by the equatorial upwelling, indicating a major response to the enhanced inorganic nutrient supply. This is consistent with reports of increased growth and abundance of *Synechococcus* with increased nitrate availability (Waterbury et al. 1986, Glover et al. 1998, Partensky 1999, Agawin et al. 2000). The increase in the abundance of cells and in the %LC for *Prochlorococcus* in this area was moderate in comparison, agreeing with the reported inability of *Prochlorococcus* to use nitrate (Moore et al. 2002).

Although *Prochlorococcus* and *Synechococcus* coexist in the Central Atlantic Ocean, their contrasting vertical distributions of dead cells indicate that these groups occupy different niches (Ferris & Palenik 1998). Both picocyanobacteria groups showed the highest fraction of dead cells at depths receiving the highest irradiance (>40% light), but *Synechococcus* showed a greater resistance to high light and a lesser capacity to survive at low irradiance than *Prochlorococcus*. These 2 different niches, evidenced by the contrasting patterns of cell viability, help explain the differences reported in the distribution of the 2 cyanobacteria groups across the Central Atlantic Ocean (Li 1995, Partensky et al. 1996, Zubkov et al. 2000, N. Agawin & S. Agustí unpubl.). The maximum *Prochlorococcus* abundance is often located in deeper waters than that of *Synechococcus*, where *Synechococcus* abundance is strongly reduced (Li 1995, Zubkov et al. 2000, N. Agawin & S. Agustí unpubl.). The presence of 2 peaks in *Synechococcus* and *Prochlorococcus* viability in the South Atlantic suggests the existence of high-light and low-light adapted cells, segregated within the water column of the SASG. Physiological adaptation to the light environment by picocyanobacteria and the presence of different ecotypes within the water column has been suggested to be a mechanism that allows survival of populations growing under broader environmental conditions, such as occur in the water column of the stratified ocean (Ferris & Palenik 1998, Moore et al. 1998). In addition to affecting the vertical variability in cyanobacteria %LC, the changes in light penetration across the different areas of the Central Atlantic Ocean should also influence its horizontal distribution. The higher picocyanobacteria %LC observed in the North Atlantic could also be related to the decrease in light penetration in this area (associated with the plankton response to the upwelling), resulting in a less stressful light environment at the surface waters.

The results indicate nutrient deficiency and high irradiance to be the main causes of picocyanobacteria cell death in the Central Atlantic Ocean. High irradiance, particularly UV irradiance, has been previously reported to stress phytoplankton cells (Vincent & Roy 1993, Berges & Falkowski 1998, Boelen et al. 2000). Besides light and nutrient availability, other causes of phytoplankton cell death should be not neglected as potential factors influencing the viability of picocyanobacteria cells. Virus infection has been reported to be responsible for the death of an important proportion of cyanobacteria in the ocean (e.g. Proctor & Fuhrman 1990). Also, Mann et al. (2002) recently identified copper toxicity as a factor able to influence *Prochlorococcus* and *Synechococcus* distribution in the Sargasso Sea.

Whatever the factors influencing picocyanobacteria cell death in the Central Atlantic Ocean, the greater fraction of dead cells observed for *Prochlorococcus* indicates that cell death, as a major source of losses, should be especially important for the dynamics of this organism in the Atlantic Ocean.

Picocyanobacteria cell death was detected here using a cell membrane permeability test (Darzynkiewicz et al. 1994, Agustí & Sánchez 2002). The increase in cell membrane permeability is considered a key event (i.e. a point of no return) in the progression of cell death processes, and characterizes both necrotic and advanced apoptotic cells (Wyllie et al. 1980, Ellis et al. 1991). Once a cell loses the ability to maintain homeostasis, the cellular compounds are unavoidably excreted into the medium (e.g. Myklestad 2000). In addition to the consequences of cell death for population dynamics, the increased membrane permeability of the dead *Synechococcus* and *Prochlorococcus* cells detected here should lead to the release of dissolved cellular compounds into the surface waters of the Central Atlantic Ocean. The direct consequences for the carbon flux remain to be quantified.

In summary, the %LC for picocyanobacteria was low in the oligotrophic Central Atlantic Ocean, indicating that cell death must be an important process regulating picocyanobacteria dynamics. In addition, the high fraction of living picocyanobacteria cells in waters affected by the equatorial upwelling, and the low fraction of living cells in the ultraoligotrophic SASG, suggest a relationship between phytoplankton cell mortality and the trophic conditions of the water. The results also show a prevalence of dead cells in waters receiving high irradiance, suggesting that high light intensity is an important

source of mortality in these oligotrophic waters. The results identify important differences in the distribution of living cells of *Synechococcus* and *Prochlorococcus*, with *Synechococcus* being more resistant to moderate light, and *Prochlorococcus* being more resistant to low light intensities. This suggests that differential sensitivity to stress factors may contribute to niche segregation of the 2 picocyanobacteria groups in the Atlantic Ocean. This study identifies cell death as a process structuring phytoplankton communities, which is likely to affect the carbon flow in the Central Atlantic Ocean.

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